Abstract

Vernolic acid (*cis*-12,13-epoxy-*cis*-9-octadecenoic acid) occurs as the triglyceride in the seed of *Vernonia anthelmintica*. Incubation of the seed produces a 1,3-divernolin. To determine whether the structure of trivernolin is responsible for the apparent secondary ester position specificity of the natural enzyme, trivernolin and triolein, were incubated with pancreatic lipase and the free fatty acids and monoglycerides were determined after 5 and 15 min digestion periods. The preponderance of 2-monoglyceride produced by the action of pancreatic lipase was interpreted to indicate that the structure of trivernolin was not solely responsible for the secondary position specificity of the *V. anthelmintica* lipase toward trivernolin.

Introduction

TRIVERNOLIN, a simple triglyceride of vernolic acid was found by Krewson et al. (6) as a major constituent of the oil from the seed of *Vernonia anthelmintica*. There was present a hydrolytic enzyme system in the seed which converted trivernolin to 1,3-divernolin and vernolic acid with no apparent formation of monoglyceride. (6) This was of interest in that most lipases are either specific for the primary esters of glycerides, or do not exhibit any positional specificity (5,8,10,12).

If the lipase system of *V. anthelmintica* is specific for the secondary esters of other triglycerides, it would be most useful in determining triglyceride structure. But before pursuing such investigations, it is necessary to know whether the specificity is due to the action of the lipase, or to the epoxy structure of trivernolin. This investigation was undertaken to partially test this hypothesis by incubation of purified trivernolin and pancreatic lipase of known specificity (10,12) and comparing free fatty acid and monoglyceride contents of this system with one of pancreatic lipase and triolein.

Materials and Methods

Purified trivernolin was prepared at the Eastern Regional Research Laboratory from *V. anthelmintica* seed oil by a process of crystallization and column chromatography (7). Commercial triolein (Hormel) was used as purchased. These triglycerides were checked for purity by thin layer chromatography (TLC) using 75:25 ethyl ether and petroleum ether (30–60C) as the developing solvent and a brom-thymol-blue spray (9) to visualize the spots. In the solvent system used, triglycerides travel just below the solvent front. Both substrates gave only one spot and that was in the area of triglycerides.

A crude preparation of pancreatic lipase (Steapsin, Fisher Scientific) was treated four times to remove ca. 10% lipid materials as follows: 2 g of the crude powder was slurried in 200 ml ethyl ether, stirred for 15 min magnetically and the mixture centrifuged; the ether layer was decanted and after the final treatment, the wet powder was dried at room temp over calcium sulfate in a desiccator. Prior to each digestion trial, the enzyme was dispersed by mixing either 30 or 80 mg with 1 ml of 1 M tris buffer (pH 8.0). The digest consisting of 200 mg of substrate, 10 ml of 10% gum arabic in 1 M tris buffer (pH 8.0), 0.2 ml of a 1% bile salt solution (Difco), and 0.5 ml of a 45% calcium chloride solution (11), was mixed in a Waring Blendor. To this, 1 ml of the lipase dispersion was added and the contents incubated at 37C with shaking. Controls were similarly prepared except that no enzyme was added. Eight trials were run with trivernolin and two with triolein each for 5 and 15 min digestion periods. Two additional samples using 80 mg of pancreatic lipase were run at both of these digestion periods with each substrate. Immediately after incubation the mixture was placed in a casserole, acidified with 0.5 ml 20% sulfuric acid, and the lipids extracted in the following manner. The reagents and apparatus are described by Harper et al. (1), except that 90:10 chloroform-methanol (v/v) was used as the solvent. Fifty g activated silicic acid was added to the contents of the casserole and the mixture was ground with a pestle. To this, 50 ml of chloroform-methanol solvent was added with additional grinding, and the resulting slurry was poured into a chromatographic column fitted to a suction flask. An additional 150 ml of solvent was used to rinse the casserole and elute the lipids from the column. The extraction was facilitated by vacuum from a water aspirator.

Half of the extract from each sample was analyzed for free fatty acids by titration to the thymol blue end-point with 0.05*N* alcoholic KOH. The solvent was removed from the second half with a rotary flash evaporator, and the lipid material was dried in a desiccator under vacuum. The contents were then diluted to 50 ml with chloroform and duplicate 1 ml aliquots were analyzed for monoglycerides. The monoglycerides were estimated by the method of Jensen and Morgan (2) in which the formaldehyde produced by periodic acid oxidation is measured colorimetrically by reaction with chromotropic acid. In this procedure the chloroform-monoglyceride mixture is analyzed before and after perchloric acid isomerization to determine one and total monoglycerides.

Results and Discussion

The free fatty acid and monoglyceride contents are given in Table I; also the data for the diglyceride products which were calculated from the free fatty acid and monoglyceride contents with the assumption that glycerol was not formed in these digestions (3, 10). The free fatty acid and monoglyceride values obtained from trivernolin were somewhat lower than those obtained for triolein under the same digestion conditions, indicating that the rate of hydrolysis was different for the two glycerides. However, it should be noted that the difference occurred within the first 5 min of digestion. If the 5-min values are subtracted

[1] E. Utiliz. Res. & Dev. Div., ARS, U.S.D.A.

from the 15-min values, it is clear that the rates of digestion were nearly identical for the last 10 min. Similar calculations can be made for the 80 mg pancreatic lipase samples and yield the same results, viz., that the difference in rate of hydrolysis between triolein and trivernolin is a difference in the initial rate of hydrolysis.

The 2-monoglyceride present as per cent of total monoglyceride formed was comparable for the two substrates and illustrates the similar behavior of these substrates during lipolysis by pancreatic lipase. The presence of 1-monoglyceride in the digestion products was probably due to acyl migration (11), although 2-position hydrolysis cannot be ruled out. The lower percentage of 2-monoglyceride after the 15 min digestion periods has been observed (4) and can be interpreted as increased acyl migration to the 1-isomer. The preponderance of 2-monoglyceride supports the concept of pancreatic lipase specificity for the primary ester position since this was observed for both triolein and trivernolin. Thus trivernolin acts as most triglycerides and does not possess peculiar behavior due to the epoxy groups. Although final proof awaits the incubation of other triglycerides with the lipase of *V. anthelmintica*, the findings of Krewson et al. (6) can now be studies in terms of an enzyme system that may be specific for the internal position of trivernolin and other triglycerides.

An interesting observation during this study concerns the calculated amounts of diglyceride. Whether trivernolin or triolein was used, the amount of diglyceride present after 15-min digestion periods was the same as that present after 5 min for similar amounts of enzyme. An increase in free fatty acid and an increase in monoglyceride with no increase in diglyceride content implies that diglyceride was being converted to monoglyceride as quickly as triglyceride was being hydrolyzed to diglyceride, at least during the last 10 min. In contrast, we have noted recently (4) that pancreatic lipase did not hydrolyze 1,3-dilaurin as rapidly as it hydrolyzed trilaurin. Although it is possible, under these conditions, that diolein may be digested as rapidly as triolein, it is tempting to ascribe the diglyceride values in this study to the enzyme-substrate complex. If the enzyme, once attached to a triglyceride, is released only when a monoglyceride is formed, the diglyceride content would remain constant until all available triglyceride substrate is gone.

TABLE I

Products of Pancreatic Hydrolysis of Triolein and Trivernolin (except where otherwise indicated all figures are in microequivalents)

Product	30 mg pancreatic lipase/sample				80 mg pancreatic lipase/sample			
	5-min digest		15-min digest		5-min digest		15-min digest	
	TV	TO	TV	TO	TV	TO	TV	TO
FFA	163	192	228	253	196	265	308	368
Total MG	39	55	73	85	50	83	103	139
1–MG	10	16	26	34	9	24	25	42
2–MG [a]	29	39	47	51	41	59	78	97
DG [b]	85	82	82	83	96	99	102	90
% 2–MG	74	71	64	60	82	71	76	70

TV = Trivernolin; TO = Triolein; FFA = Free Fatty Acid; MG = Monoglyceride; DG = Diglyceride.

[a] Calculated by difference (Total MG — 1–MG).

[b] Calculated from FFA and MG—assuming no glycerol was produced [FFA — 2(Total MG)].

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